EVALUATION OF ANTI CANCER ACTIVITY OF *NIGELLA SATIVA* STEM EXTRACT BY INHIBITION OF CELL PROLIFERATION

A Dissertation submitted to
THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY,
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In partial fulfillment of the requirements for the award of the Degree of
MASTER OF PHARMACY
IN
BRANCH –VI - PHARMACOLOGY

Submitted by
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MAY - 2017

CERTIFICATE

This is to certify that the dissertation entitled **"Evaluation of anti cancer activity of *Nigella Sativa* stem extract by inhibition of cell proliferation"**being submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai was carried out by **Ms. AMRUTHA** to The Tamil Nadu Dr.M.G.R Medical University, Chennai in partial fulfillment for the degree of **MASTER OF PHARMACY IN PHARMACOLOGY** is a bonafied work carried out by candidate under my guidance and supervision in the Department of Pharmacology , Karpagam college of Pharmacy Coimbatore – 32.

I have fully satisfied with her performance and work. I have forwarded this dissertation work for evaluation.

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Principal

DECLARATION

I hereby declare that this dissertation **"Evaluation of Anti cancer activity of *Nigella Sativa* stem extract by inhibition of cell proliferation"** submitted by me , in partial fulfillment of requirements for the degree of MASTER OF PHARMACY IN PHARMACOLOGY to The Tamil Nadu Dr.M.G.R Medical University, Chennai is the result of my original and independent research work carried out under the guidance of **Prof. G. NAGARAJA PERUMAL., M.Pharm.,** Professor & Head Department of Pharmacology ,Karpagam College of Pharmacy, Coimbatore -32.

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EVALUATION CERTIFICATE

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Examination centre:

Date:

Internal Examiner Convenor of Examination

External examiner

DEDICATED TO MY BELOVED PARENTS ,

SIBLINGS ,TEACHERS

,FRIENDS AND

ALMIGHTY

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AMRUTHA P

(261526157)

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ABBREVIATIONS

- K RAS (k-ras) : V- Ki- ras 2 kirsten rat Sarcoma viral oncogene homologue
- CRC – Colo rectal Carcinogenesis
- CTNNB1- Catenin Beta 1
- PIK3CA - Phosphatidyl Inositol – 4,5 Bi phosphate 3- Kinase catalytic Sub unit Alpha
- APC – Adenomatous polyposis
- TP 53 – Tumour Protein 53
- DCC – Deleted in Colo rectal carcinoma
- Wnt – A group of signal transduction made of proteins
- CIMR – Chromosomal Instability in m RNA
- DMH – Di Methyl Hydrazine
- AOM – Azoxy Methane
- MAM – Methyl Diazonium Ion
- MTT – 3-(4,5 Di methyl thiazole2-Yl)-2,5- Diphenyl tetrazolium bromide

CHAPTER I

1. INTRODUCTION

1.1 Cancer

Cancer is most lifethretening diseases which are characterized by uncontrolled cellular growth of abnormal cells anywhere in the body. Normally body forms new cells as it need cells to replace old cells which may die. Cancer make this go wrong. Cancer make new cells even when body doesn't require it and the old won't die.[1]

Carcinogenesis or oncogenesis or tumouriogenesis is the formation of cancer whereby normal cells are transformed to cancer cells. The process is characterised by changes at cellular genetic and epigenetic levels and abnormal cell division sometimes forming malignant mass. The different phases of carcinogenesis is a multi-step process that includes initiation phase, promotion phase, conversion phase and finally progression phase. The growth of both normal and cancerous cells is genetically controlled by the balance or imbalance of oncogene, proto oncogene and tumor suppressor gene. Multiple genetic mutations are required to convert normal cells to cancerous cells. Apoptosis and cellular senescence (aging) are normal mechanisms for cell death. Cancer cells are genetically unstable, which results in tumor masses of heterogeneous cells and makes the cancer a "moving target" for drug therapy.[2]

Cancer treatment include cure, prolongation of life and relief of symptoms. Surgery and radiation therapy are the best chance of cure for patients with localized cancers, but systemic treatment methods are required for systemic cancers. But main impacts include long-term complications, such as infertility, secondary malignancies, effects on physical or intellectual development and major organ damage, can negatively affect health and quality of life for cancer survivors.

Induction of apoptosis is one of the most important markers of cytotoxic antitumor agents. Some natural compounds including plants induce apoptotic pathways that are blocked in cancer cells through various mechanisms in cancer cells.

1.2. Importance of Herbal treatment and remedies against cancer

The term “cancer” was used first time by the Hippocrates, father of western medicine, Derived from Greek words “carcinoma” and “Karakinos” to describe tumor.

Multiple surveys reported that people with cancer commonly use herbs or herbal products. Vinca Alkaloids, Texans, podo phyllotoxin, Camptothecins have been clinically used as Plant derived anticancer agents.[3]

Plant derived anti cancer products introduced during 1981-2002, 48 drugs natural products includes

- vinca alkaloids (vinblastine, vincristine, vindesine, vinorelbine)
- taxanes (paclitaxel, docetaxel)
- podophyllotoxin and its derivations (topothecan, irinothecan)
- Anthra cyclines (doxorubicin, daunorubicin, epirubicin, idarubicin)

With the invention of vincristine, vinblastine and vinca alkaloid and cytotoxic podo phyllotoxin, the study of herbal sources for cancer treatment started extremly in 1959. These all lead National Cancer Institute to start a program called plant collect in 1960. These findings resulted in discovery of novel chemotypes with cytotoxic activities including the taxans, camptothecins.

Even from the history natural products have afforded as a rich source of compound with many medicinal applications. Ancient Unani manuscripts Egyptian papyrus and Chinese writings described the use of herbs. Evidence exist that Unani Hakims, Indian Vaids and European and Mediterranean cultures were using herbs for over 4000 years as medicine. Indigenous cultures such as Rome, Egypt, Iran, Africa and America used herbs in their healing rituals, while other developed traditional medical systems such as Unani, Ayurveda and Chinese Medicine in which herbal therapies were used systematically.[4][5]

Traditional systems of medicine continue to be widely practised on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. Being an Indian especially we have to be proud about many natural methods of treatment including the important Ayurvedic system of medicine. Where we could find many effetive herbal formulations and still growing field of medicine.

So it has become a responsibility as well as competition in the field of research to find plant constituents against the major threatening disease cancer. Cancer is considered as the major cause of death similiar to myocardial infraction. Millions die and at same time suffering the after effects and impacts of cancer. Last century was able to perform great advances in modern medicinal science to control diseases. But many diseases like cancer are not yet curable fully. Though all are approaching Allopathic system of medicine mostly traditional or folk system of medicine are runnnig parallel.[6]

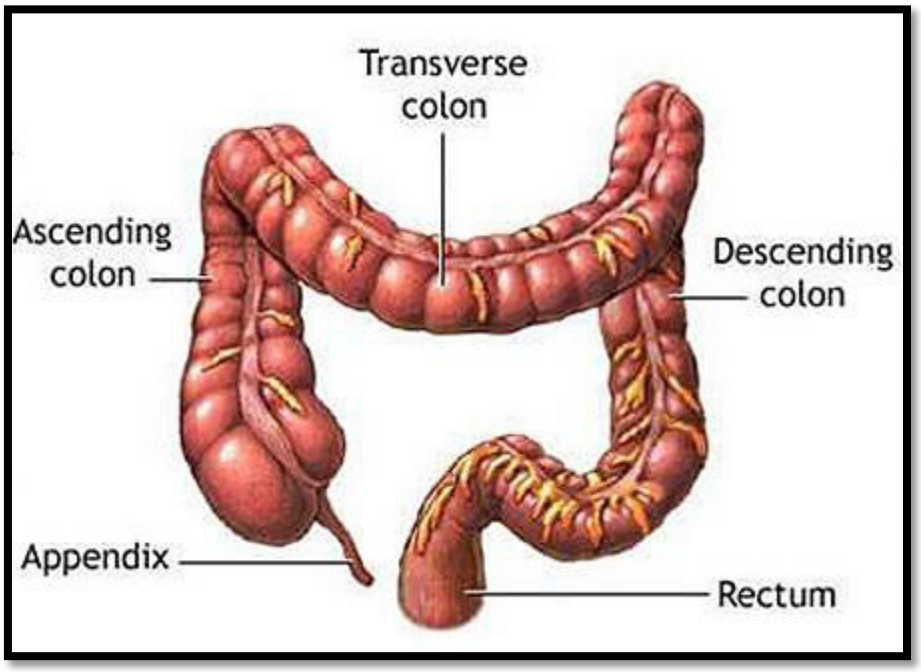
Not only curing has become trend all are ready to follow the word “prevention is better than cure”. Nowdays awareness programmes and precautious measures against cancer are going well. Where herbal formulations find the way of it’s importance as preventive measures.

The plant *Nigella sativa* seed has been an important nutritional flavouring agent and natural remedy for many ailments for centuries in ancient system of medicine including Unani,Ayurveda Chinese and Arabic medicines[7]. Traditionally, there is a common Islamic belief that blackseed is a panacea for all ailments,but cannot prevent aging or death. It originated from Southeastern Asia and also used in ancient Egypt, Greece, Middle east and Africa. Colon and rectal cancer is one of the common cancers among men and women from the India as per recent reports. It ranks sixth or seventh among all other cancers. The incidence (newly diagnosed cases of cancer in a year) of colon and rectal cancers in India is about 4 patients per 1,00,000 population for both sexes together. [8]

1.3.Colon

The colon is another term for the large intestine, it is the lowest part of the digestive system. Inside the colon, water and salt from solid wastes are extracted before the waste moves through the rectum and exits the body through the anus.

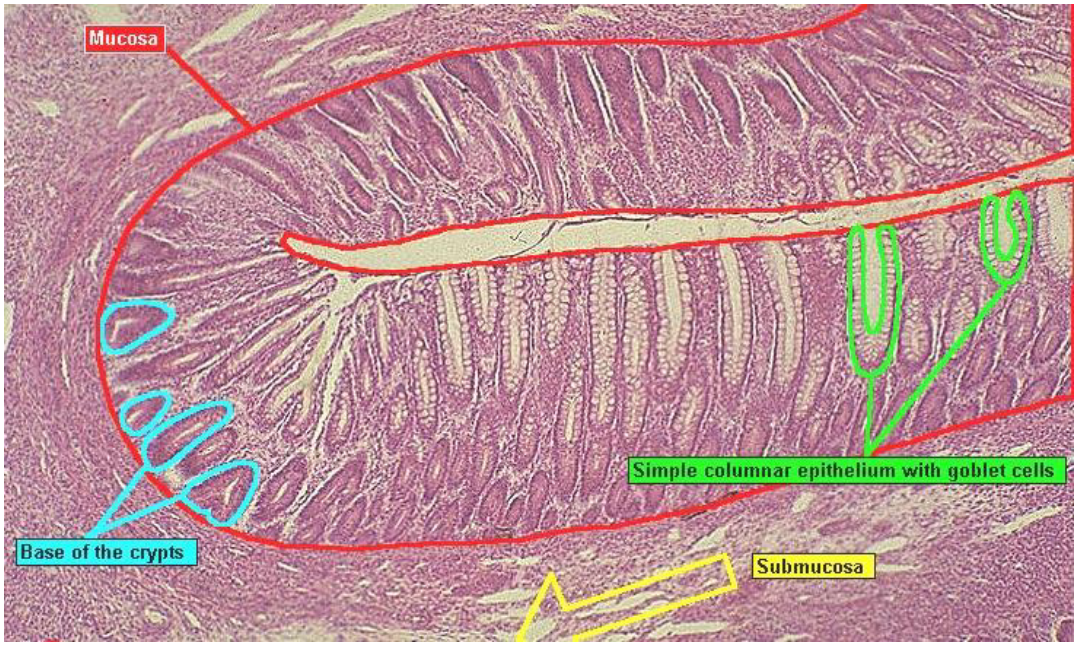
Fig.No: 1: Anatomy of colon



The colon is made of several sections. The ascending colon travels up the right side of the abdomen. The part that sits across the abdomen is called the transverse colon. The descending colon runs down the left side of the abdomen. The next segment, the sigmoid colon, joins the descending colon to the rectum. Stool leaves the body through an opening called the anus.

1.3.1.Histology of Colon

Fig. No: 2: Histology of colon

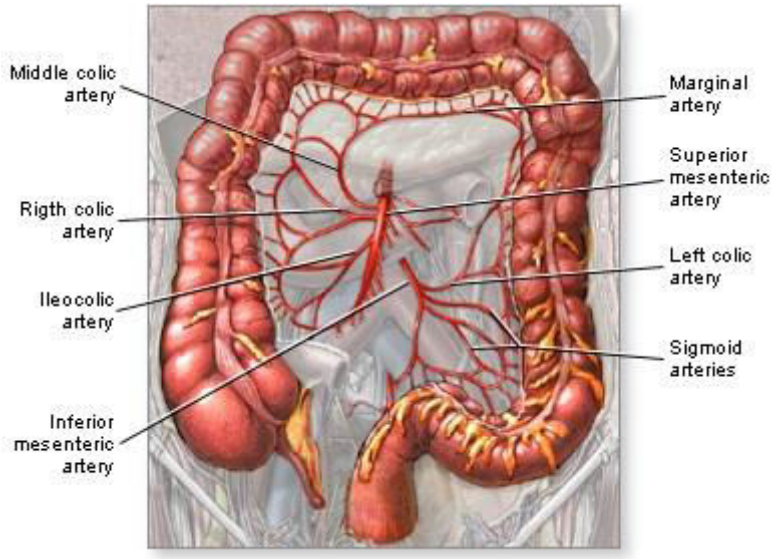


- It have mucosa which have epithelium, intestinal glands(Glands of Lieberkuhn), Lamina prpopria and muscularis mucosa.
- A submucosa which muscularis externa containing inner circular and outer longituidinal smooth muscle layers.
- The outermost serosa
- Lymphatic nodules in the lamina propria and submucosa.
- A myentric (Auerbach)nerve plexus(Parasympathomimetic)exists between the muscularis externa layer.

1.3.2 Blood supply to colon

The colon is supplied with the superior mesenteric artery(SMA),the inferior mesenteric artery (IMA), and branches of the internal iliac arteries.

Fig. No 3: Blood supply to colon

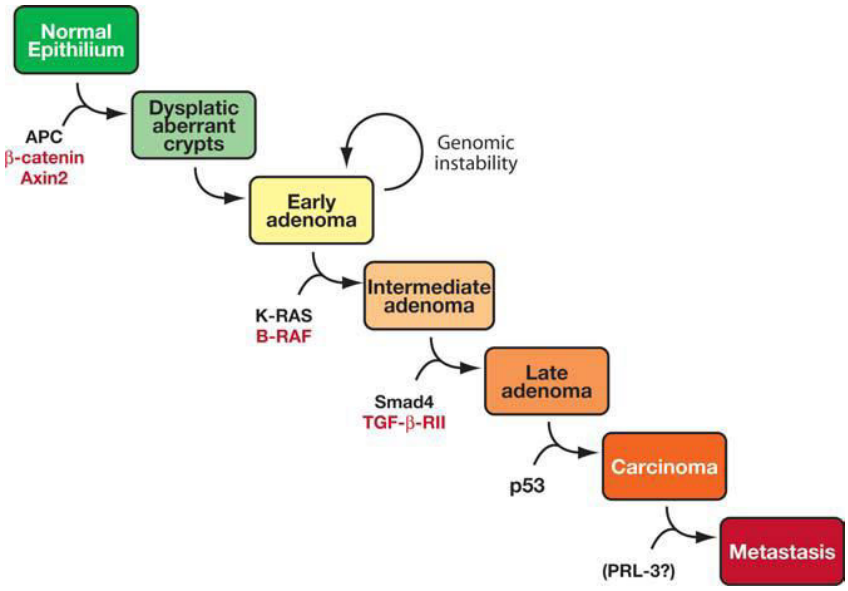


- The SMA branches to the middle idic right colic and ileocolic arteries which supply the right colon and the right half of the transverse colon.
- The IMA gives rise into left colic sigmoid and superior rectal hemmorhoids areteries.
- The distal rectum is supplied by inferior and middle rectal (hemmorhoi-dal)arteries which are branches of internal iliac artery.
- There is an extensive mesenteric collateral circulation that provides substantial protection from ischemic insults.
- The SMA and IMA communicate through the marginal artery of Drum-mond which runs along the splenic flexure and the arc of Riolan.
- The marginal artery of Drummond absent or underdeveloped in 5% population.

1.4.Colon Cancer

Cancer is a class of disease characterised by the uncontrolled cell growth.Colon cancer forms when the same happens. Depending on the type of cancer, 5 to 20 years may elapse between the carcinogenic phases and the development of a clinically detectable cancer. The final stage of neoplastic growth called as progression, which involves further genetic changes leading to increased. Most colon cancers originate from small non cancerous tumours adenomatous polyps that forms the inner walls of large intestine. Even colon polyps if not removed may lead to colon cancers have reported. Colon cancer cells will invade to other healthy tissue that is near to the tumour causing further complications. Incidence of colon cancer is higher in developed countries[9]. But in India it was reported in low rate which now is going up with modern life style.[10]

Fig.No 4: Correlation between CRC Progression and the accumulation of genetic alterations



1.4.1.Pathology Of Colon Cancer

Researchers have studied tha mechanism behind colon cancer formation significantly. And also it's development. The existence of of major three pathways are reported.Chromosomal instability microstellite instability and CpG island methylator phenotype. Other important one study recently reported that several genetic and epigenetic changes are important in determining patients survival probability.

Several epidemiological studies have confirmed the involvement of numerous environmental and dietary factors, such as cigarette smoking, alcohol abuse, a diet high in fat and low in fiber, a sedentary lifestyle and obesity .

Colorectal cancer (CRC) is the second most common cancer in women and the third most common in men globally. CRC arises from one or a combination of chromosomal instability, CpG island methylator phenotype, and microsatellite instability. [11]

Table No.1: Overall Prevalence of Genetic Mutations in CIN-positive Colo-rectal Cancers

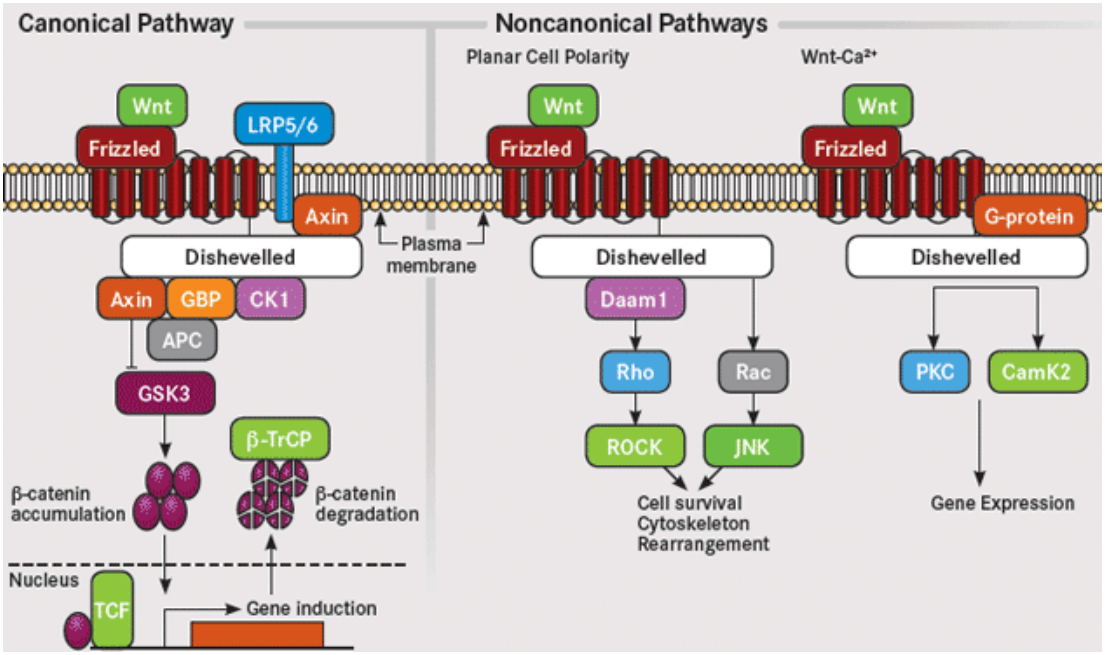
Reported by US national library of medicine [11]

Gene	Chromosomal location	Prevalence of mutation	Function of product
Oncogene			
KRAS	12p12	~30-50%	Cell proliferation,survival
CTNNB1	3p22	~4-15%	Regulation of Wnt pathway
PIK3CA	3q26	~20%	Cell proliferation, survival
Tumour suppressor genes			
APC	5q21	~30-70%	Inhibition of Wnt signalling
TP53	17p13	~40-50%	Cell cycle arrest, apoptosis
SMAD4,SMAD2	18q21	~10-20-%	Intracellular mediators of TG – F-β Pathway
DCC	18q21	~6%	Cell surface receptor for netrin-1

1.4.1.1.Chromosome instability pathways

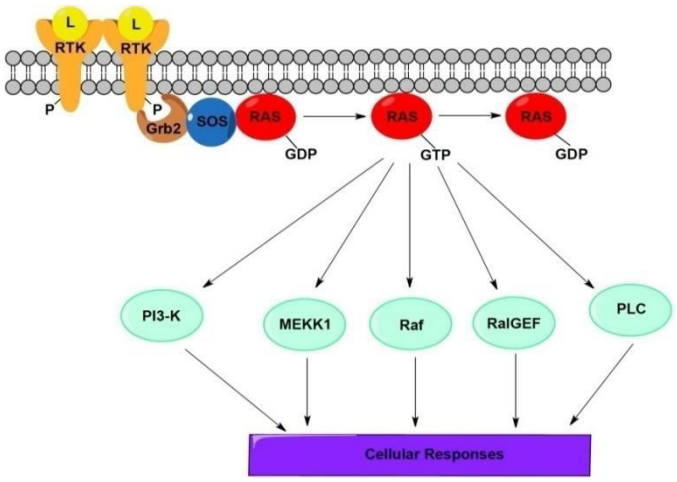
"Wnt signalling" : Here the tumuriogenic process involves different mitotic spindle check point regulators and proteins that mutually influence mitotic chromosome stability.Which is charecterised by the early adenomatous polyposis . Wnt involves pathways made of proteins pass signals into a cell through cell surface receptors.[12]

Fig. No:5: Chromosome Instability Pathways



1.4.1.2.RAS pathway: The adenoma to carcinoma transition is regulated initially by the *K-ras* gene, a proto-oncogene that encodes for the GTPase protein involved in the transduction and propagation of extracellular signals—e.g., mitogen-activated protein kinase (MAPKs). Mutations of *K-ras* lead to a permanently active state that permits the cell to evade apoptosis and acquire a growth advantage.

Fig.No: 6: RAS Pathway



1.4.1.3. P 53 system:

p53 loss of function is frequently present in the later stages of colorectal tumorigenesis. The *p53* gene is located on chromosome 17p and its mutation is one of the key steps in colorectal carcinogenesis and stimulates high proliferative activity through the loss of cell cycle control and apoptosis.

1.4.1.5.Other pathways:

An often co-occurring molecular alteration with the *p53* loss is the LOH of chromosome *18q* (65.4%) , where the genes *Smad2*, *Smad4* and *DCC* genes are located. LOH of *18q* has been associated with a strong negative prognosis in colon cancer, in particular with high metastatic potential.

Microsatellite instability:

Which depicts the form of instability in case of sporadic colorectal cancer (15%)and 95% in case of herditary non polyposis syndrome.[13]

CIMR (chromosomal instability in m RNA) and "serrated " pathway

Here the mechanism is hyper methylation of CpG dinucleotide sequences localized in promotor regions of genes involved in cell cycle regulation, apoptosis, angiogenesis, DNA repair, invason and adhesion. And thereby observed loss of gene expression.

1.4.2.Symptoms Of Colon Cancer[14]

- Changes in stool consistency
- Diarrhoea or constipation
- Narrow stools
- Rectal bleeding or blood in the stool
- Pain, cramps, or gas in the abdomen
- Pain during bowel movements
- Continual urges to defecate
- Weakness or fatigue
- Unexplained weight loss
- Irritable bowel syndrome (IBS)
- Iron deficiency.

1.4.3.Treatment Of Colon Cancer

Stage "0"of colon cancer- Here it appears as polyps. And polypectomy can be performed.

Stage "1" colon cancer -Colon cancer layers of colon wall hre. But they have not spread out of the wall.Here treatment is Partial colectomy.

Stage "2" of colon cancer- Stage where cancers have grown through wall of colon to nearby tissues but have not yet spreded to lymph nodes.Can treat by surgery along with lymph nodes.

Sometimes adjuvant chemotherapy is recommended if

- Cancer looks very abnormal
- Has grown to lymph and blood vessels
- Cancer have blocked colon
- Perforation in colon wall

Sometimes radiation therapy is given to kill remaining cancer cells if present.

Stage "3"of colon cancer- Here cancer have grown to nearby lymph nodes .Surgery and chemotherapy is recommended.

Stage "4" of colon cancer- Have spread to other organs from colon.Sometimes to liver, lungs, or distant lymph. Here chemotherapy before and after surgery is recommended.

The main drugs used in the adjuvant treatment of colon cancer are a combination of the following:

• 5-fluorouracil (5FU), which is often given with the vitamin folinic acid (leucovorin)
• capecitabine
• oxaliplatin
• irinotecan
• raltitrexed
• mitomycin C.

The most commonly-used chemotherapy drugs for advanced bowel cancer are:

- Bevacizumab
  Used to treat many types of cancers and a specific eye disease. Blocks endothelial cell proliferation and new vessel formation by inhibition of VEGF interaction on surface of endothelial cells.
- Irinotecan Hydrochloride
  Inhibit DNA replication and transcription
- Capecitabine:
  It is a prodrug of 5-FU absorbed from intestine.
- Cetuximab:
  Thymidine synthase inhibitor hence by inhibitng thymidine monophosphate, which is the active form of thymidine required for de novo synthesis of DNA.

- Ramucirumab

  It is directed against the Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) as an antagonist for blocking the growth factor binding on Receptor
- Oxaliplatin

  Formation of Platinum DNA adducts for blocking DNA replication and are cytotoxic than cisplatin
- Cetuximab

  Expression of (EGFR) being reported in cancer of colon rectum head and neck, cetuximab bind specifically on EGFR on both normal and tumour cells.
- 5-FU

  Thymidine synthase inhibitor hence by inhibitng thymidine monophosphate, which is the active form of thymidine which is a nucleoside required for DNA replication.
- Leucovorin Calcium

  As an adjuvant to get rid of the blood cell disorders due to cytotoxic drugs
- Trifluridine

  Nucleic acid synthase inhibitor
- Panitumumab

  Binding to EGFR and thereyby inhibits binding of all ligands in EGF

1.4.3.1.Side Effects

Chemotherapy can temporarily reduce the production of white blood cells in your bone marrow, making you more prone to infection. This effect can begin about seven days after treatment has been given, and your resistance to infection usually reaches its lowest point about 10–14 days after chemotherapy.
Bruising or bleeding
Anaemia (low number of red blood cells) Chemotherapy can reduce the number of red blood cells, which carry oxygen around the body. A low red blood cell count is called anaemia.
Tiredness (fatigue).
Nausea and vomiting

Diarrhoea

Sore throat

Hair loss

Numbness or tingling in hand or feet

1.5.DMH (Di Methyl Hydrazine) As Colon Carcinogen

The colon carcinogen 1,2-dimethylhydrazine (DMH) has been widely used to study chemically induced colon cancer. Whatever the mode of administration, DMH specifically induces tumours within the descending colon of rat and the histopathology is found to be similar to that observed for human sporadic colon tumours[15]

DMH mechanism is alkylating DNA and the pro-mutagenic lesion O6-methylguanine (O6-MeG) has been detected in DNA from various rat and mouse tissues following exposure to DMH.

Nowdays DMH induced colon cancer model represents useful research tool for the studies of colon carcinogens and chemopreventive agents. DMH and its metabolite AOM are the agents widely used in experimental models colorectal carcinogenesis in rodents. They are highly specific indirect colorectal carcinogens that induce the initiation and promotion steps of colorectal carcinogenesis yielding colorectal tumour lesions in a dose-dependent manner in rats, mice and hamsters.

1.5.1.Chemical Structure of DMH

H3C- NH- NH- CH3

1.5.2.Dimethyl Hydrazine metabolism

DMH is highly specific colorectal carcinogen[16] that will be metabolically activated by liver with a series of reactions through intermediates azomethane,AOM and methylazoxymethanol(MAM) to the ultimate carcinogenic metabolite, highly reactive methyldiazonium ion. 13,MAM is excreted into the bile and transported to the colon or enter directly into epithelial cells of the colon from the blood circulation. Some studies have demonstrated that rat colon epithelial cells are capable of metabolising DMH into carcinogenic metabolite

without previous metabolism by other tissues or colon bacteria. Although intestinal flora and bile acids have influence on the incidence of tumours. DMH induced colon cancer is a multistep process involving a series of pathological alterations, such as formation of aberrant cryptic foci (discrete microscopic lesion; Ionov et al., 1993**).**[17]

It was already reported about the ove expression of inducible nitric oxide synthetase and cox-2 in colorectal cancer [18]

1.5.3.Tumour lesions induced by DMH

DMH is highly specific for colonic epithelium and induces tumours mostly in large bowel. Colon specific susceptibility for this carcinogen is a result of a delayed or incomplete repair of damaged DNA in the colon compared to other organs leading to accumulation of mutations, and in a small proportion of cells giving rise to CRC.

1.6.Colorectal Tumour Lesions

Aberrant crypt foci

The first specific morphologically identifiable lesions for colonic carcinogenesis are aberrant crypt foci (ACF). They can be identified in the colon of carcinogen treated mice by the light microscopic examination of the mucosal surface of colons that had been stained with methylene blue. ACF are can be distinguished from normal crypts by their darker staining and larger size, elliptical shape, thicker epithelial lining and larger perycriptal zone.

Histologically, colorectal epithelial tumours are divided into adenomas and carcinomas. Tumours that penetrate through the muscularismucosa into the submucosa are classified as carcinomas. Adenoma is a type of non-cancerous tumor or benign that may affect various organs. It is derived from the word “adeno” meaning 'pertaining to a gland'. Adenomas are characterized by hypercellularity with enlarged, hyperchromatic nuclei,varying degrees of nuclear stratification, loss of polarity and decreased mucine excretion. ACF may appear predominantly in distal colon followed by proximal colon[19]

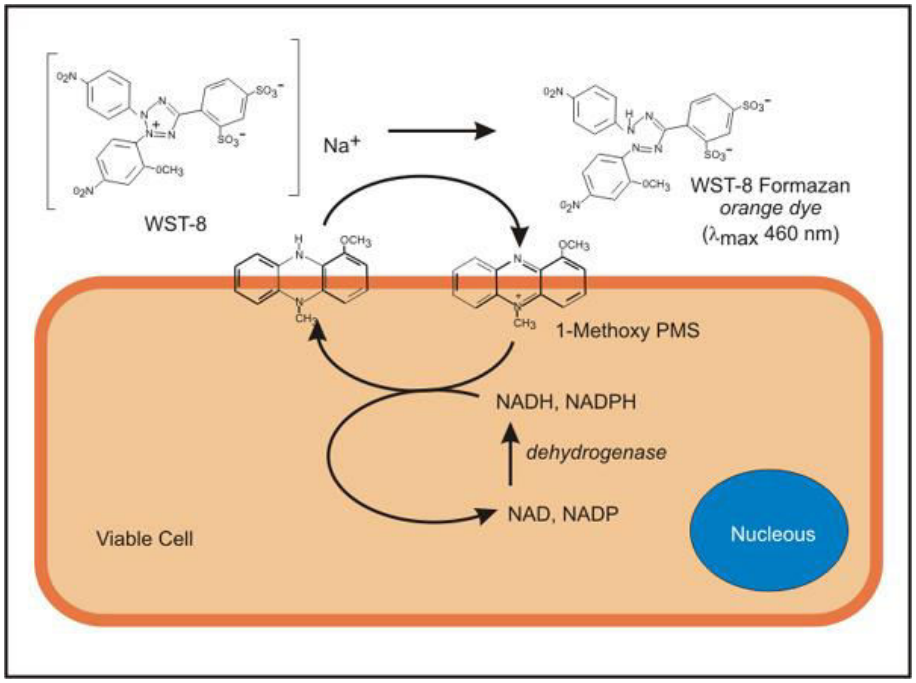
1.7.MTT Assay[20]

Principle

MTT, a yellow tetrazole, is reduced to purple formazan in living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution formed can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The degree of light absorption depends on the solvent.

MTT is membrane permeable via endocytosis and is reduced by active mitochondria in living cells. In addition, MTT was found to be membrane impermeable. Some reports suggest that MTT is taken up by cells through endocytosis and that reduced MTT formazan accumulates in the endosomal/lysosomal compartment and is then transported to the cell surface through exocytosis.

Fig. No:7: mechanism behind MTT assay



REVIEW OF LITERATURE

CHAPTER II

2.REVIEW OF LITERATURE

Osama Adnan Kensara, et al., (2016) [21] have explained that thymoquinone which is the major constituent of *Nigella Sativa* subdues tumor growth and potentiates the chemopreventive effect of 5-fluorouracil on the early stages of colorectal carcinogenesis in rats.

Riad Agbaria, et al., (2015)[22] have studied the anticancer activity of Nigella sativa and it's relation relationship with the thermal processing and quinone composition of the seed.That data indicated that controlled thermal processing of NS seeds (at 50°C–150°C) produces significantly higher anticancer activity associated with a higher thymoquinone oil content, and inhibits the NF-κB signaling pathway.

Arshad Rehmani, et al., (2014)[23] have demonstrated the therapeutic effects of *Nigella Sativa* seed and its constituents in the diseases treatment and prevention through modulation of antioxidant, anti-inflammatory, anti-tumor, hepato-protective and other genetic activities.

Amulya sridhar, et al., (2014)[24] have performed the elucidation of molecular targets of bioactive principles of *Nigella sativa* seed or black cumin relevant to its anti-tumour functionality - In this study, three different *Insilico* Reverse Screening approaches have been employed for identifying the putative molecular targets of the bioactive principles in Black cumin (thymoquinone, alpha-hederin, dithymoquinone and thymohydroquinone) relevant to its anti-tumour functionality.

Al Khalaf, et al., (2013) [25] have reviewed the anticancer and anti microbial activity of Nigella Sativa seed oil. They reviewed the anticancer activity of a thymoquinone by inhibition of NF kappa B and oncogene expression.

Randhava, et al.,(2011)[26] at University of Damam have brought a summary of all work done on Nigella sativa seed against cancer emphasizing the safety *of N.Sativa* oil when given orally. They concluded about the abun-

dant active constituent Thymoquinone and it's safety mechanism of action described earlier too.

Md Asaduzzaman Khan, et al., (**2011**)[27]have described about the anticancer actions of *Nigella sativa* seed based on it's major constituent Thymoquinone.

Effenberger, et al., (2010) [28] explained that derivatives of TQ bearing terpene-terminated 6-alkyl residues were tested in HL-60 cells and 518A2 melanoma by They found the derivatives induce apoptosis associated with DNA laddering, a decrease in mitochondrial membrane potential and a slight increase in reactive oxygen species. They tested terpene-terminated 6-alkyl residues of TQ on multidrug-resistant KB-V1/Vb1 cervical carcinoma and found the derivatives inducing cell death by apoptosis.

Boskabady, et al., (2010) [29] have explained that *Nigella Sativa* seed it has a potent antihistaminic effect on airways of asthmatic patients.

Maria P Torres, et al., (2010) [30]evaluated the down-regulatory effect of TQ on MUC4 in pancreatic cancer cells.

Nader, et al., (2010) [31]showed that the active ingredients of *N. sativa* have beneficial effects against many diseases, including cancers. For example, it is effective in the diminishing the risk of atherosclerosis by decreasing the serum low density lipoprotein cholesterol level and increasing the serum high density lipoprotein cholesterol levels.

Gawahar Shafi, et al., (2009)[32] reported that methanol, n-Hexane and chloroform extracts of *N. sativa* effectively killed HeLa (human epithelial cervical cancer) cells by inducing apoptosis.

And later it was proved that black cumin seeds have anticarcinogenic and antimutagenic properties Bourgou et al., 2008.

Kanter, et al., (2009)[33] examined that *Nigella sativa* seed exerts therapeutic and protective effect in diabetes by decreasing morphological changes and preserving pancreatic beta-cell integrity and by beneficially changing the hepatic enzyme activities.

Banerjee, et al., (2009)[34] proved about the *Nigella Sativa* seed extract which can abrogate gemcitabine- or oxaliplatin-induced activation of NF-kappa B, resulting in the chemosensitization of pancreatic tumors to conventional therapeutics. They said high molecular weight glycoprotein mucin 4 (MUC4) is aberrantly expressed in pancreatic cancer and contributes to the regulation of differentiation, proliferation, metastasis, and the chemoresistance of pancreatic cancer cells.

Pari and Sankaranarayanan, (2009)[35]; brought the evidence that *N.Sativa* seed is effective against hypertension.That study evaluated the anti-hyperglycemic potential of thymoquinone (TQ), major constituent of Nigella sativa seeds on the activities of key enzymes of carbohydrate metabolism in streptozotocin (STZ)-nicotinamide (NA)-induced diabetic rats.And a significant effect in hepatic enzyme activities and thereby Anti hyperglycaemic effect was seen

Chehl, et. al., (2009)[36] have showed that TQ, the major constituent of *N. Sativa* seed oil extract, induced apoptosis and inhibited proliferation in PDA (pancreatic ductal adenocarcinoma) cells. They also suggested TQ as a novel inhibitor of pro-inflammatory pathways, which provides a promising strategy that combines anti-inflammatory and proapoptotic modes of action.

Nagi and Almakki, (2009) [37] reported that oral administration of TQ is effective in increasing the activities of quinone reductase and glutathione transferase and makes TQ a promising prophylactic agent against chemical carcinogenesis and toxicity in hepatic cancer.

El Shenawy, et al., (2008)[38] have discussed about the protective effect of *Nigella sativa* in kidney tissue against oxygen free radicals, preventing renal dysfunction and morphological abnormalities with evidence.

Aggarwal, et al., (2008)[39] reviewed about the seed *Nigella Sativa* that it is called black cumin in English, while in old Latin it was called 'Panacea' meaning 'cure all'; in Arabic it is termed as 'Habbah Sawda' or 'Habbat el Baraka' translated as 'Seeds of blessing'. It is also known as 'Kalo jeera' (in

Bangladesh), 'Kalonji' (in India) and 'Hak Jung Chou' in (China). Both seeds and oil extracted from this plant are used in medicinal purposes.

Yi et al., (2008)[40] found that TQ blocked angiogenesis in vitro and in vivo, prevented tumor angiogenesis in a xenograft human prostate cancer (PC3) model in mouse, and inhibited human prostate tumor growth at low dosage with almost no chemotoxic side effects. Furthermore, they observed that endothelial cells were more sensitive to TQ-induced cell apoptosis, cell proliferation, and migration inhibition compared with PC3 cancer cells. TQ also inhibited vascular endothelial growth factor-induced extracellular signal-regulated kinase activation but showed no inhibitory effects on vascular endothelial growth factor receptor 2 activation.

Khattab, et al., (2007)[41]explained that Thymoquinone inhibits tumor angiogenesis and tumor growth through suppressing AKT and extracellular signal-regulated kinase signaling pathways which is the major constituent of *Nigella Sativa* seed.

Ait Mubrek, et al., (2007)[42] evaluated the invitro and in vivo anti-cancer effects of Nigella Sativa seed extracts .The essential (IC 50=2%)oil and ethyl acetate (IC50 = 0.75%) extracts were more cytotoxic against the P815 cell line than the butanol extract (IC50= 2%). Similar results were obtained with the Vero cell line. In this study a comparitive data regarding the *in vitro* cytotoxic effect of the essential oil and the ethyl acetate and butanol extracts of *N. sativa* seeds on a panel of tumor cell lines was provided.

Kaseb, et al.,(2007) [43] demonstrated that N. sativa, inhibited DNA synthesis, proliferation, and viability of cancerous (LNCaP, C4-B, DU145, and PC-3) but not non-cancerous (BPH-1) prostate epithelial cells by down-regulating AR (androgen receptor) and E2F-1 (a transcription factor.)

Xia Wang and Yong Lin, (2007) [44]have concluded that , Tumor necrosis factor (TNF) is a multifunctional cytokine that plays important roles in diverse cellular events such as cell survival, proliferation, differentiation, and death. As a pro-inflammatory cytokine, TNF is secreted by inflammatory cells, which may be involved in inflammation-associated carcinogenesis. TNF ex-

erts its biological functions through activating distinct signaling pathways such as nuclear factor κB (NF-κB) and c-Jun N-terminal kinase (JNK). NF-κB is a major cell survival signal that is anti-apoptotic while sustained JNK activation contributes to cell death.

Norwood, et al. (2006)[45] explained that how Thymoquinone as chemotherapeutic agent on SW-626 colon cancer cells, in potency, which is similar to 5-flurouracil in action. The results of this study have shown a similar significant decrease in cell number as early as 24 hours in the groups treated with TQ and EGCG compared to 5-FU. Increased cellular damage were evident after 24, 48, and 72 hours and in all treated groups compared with the control. Reduced cell numbers in the treated groups suggests the possibility that TQ and EGCG may have similar chemotherapeutic effects on cancer cells as 5-FU.

El-Mahdy, et al., (2005)[46] reported that TQ exhibits anti-proliferative effect in human myeloblastic leukemia HL-60 cells. TQ in *Nigell Sativa* exhibits antiproliferative effect, induces apoptosis, disrupts mitochondrial membrane potential and triggers the activation of caspases 8, 9 and 3 in myeloblastic leukemia HL-60 cells. The apoptosis induced by TQ was found to be inhibited by a general caspase inhibitor, z-VAD-FMK; a caspase-3-specific inhibitor, z-DEVD-FMK; as well as a caspase-8-specific inhibitor, z-IETDFMK. Moreover, the caspase-8 inhibitor blocked the TQ-induced activation of caspase-3, PARP cleavage and the release of cytochrome c from mitochondria into the cytoplasm.

Thabrew, et al., (2005),[47] investigated the cytotoxic activity of N. sativa seed was tested on the human hepatoma HepG2 cell line by and 88% inhibitory effect on HepG2 was found after 24-hr incubation with different concentrations (0–50 mg/ml) of the N. sativa extract.

El-Aziz, et al., (2005)[48] could find that *N. sativa*, in combination with melatonin and retinoic acid reduced the carcinogenic effects of DMBA (7, 12-di-methylbenz(a)anthracene) in mammary carcinoma of rats.

Awad, (2005)[49] examined that oil of N. sativa could decrease the fibrinolytic potential of the human fibrosarcoma cell line (HT1080) in vitro. In this study, Nigella sativa oil (NSO) was evaluated for it's effect on the fibrinolytic potential of the fibosarcoma cell line HT1080 to elucidate whether this oil might have an antitumor activity through its modulation of the fibrinolytic potential of such cells.

Rooney and Ryan, (2005)[50] have stated that there is no preventive role of TQ on MIA PaCa-2 (pancreas carcinoma) cells. As HEp-2 human laryngeal carcinoma cells were the most susceptible, have sought to better understand the mechanisms involved by using buthionine sulfoximine (BSO), a selective inhibitor of glutathione (GSH) synthesis, to determine the importance of GSH in the apoptosis elicited, using cisplatin as internal standard. BSO significantly enhanced alpha-hederin- and cisplatin- mediated toxicity as assessed by the MIT assay, without changes in apoptosis or necrosis levels.

Khan and Sultana, (2005)[51] reported the chemo-preventive effect of N. sativa against ferric nitrilotriacetate (Fe-NTA)-induced renal oxidative stress, hyper-proliferative response and renal carcinogenesis. Treatment of rats orally with N. sativa (50 100 mg/kg body wt) resulted in significant decrease in H2O2 generation, DNA synthesis and incidence of tumors.

Gali-Muhtasib, et al., (2004)[52] proved that ThymoQuinone is antineoplastic and pro-apoptotic against colon cancer cell line HCT116. It was shown to possess potent chemopreventive activities against DMBA-initiated TPA-promoted skin tumors in mice. Despite the potential interest in TQ as a skin antineoplastic agent, its mechanism of action has not been examined yet. Using primary mouse keratinocytes, papilloma (SP-1) and spindle (I7) carcinoma cells, they studied the cellular and molecular events involved in TQ's antineoplastic activity.

Newell and Heddle, (2004)[53]**;** have performed 1, 2 Dimethyl hydrazine (DMH), is a potent colon carcinogen, inducing colorectal tumors in experimental animals and is the most widely used model of chemically induced colon.

Ali and Blunden, (2003)[54] stated that *Nigella Sativa* seeds contain both fixed and essential oils,proteins,alkaloids, and saponin as well as much of the biological activities of the Nigella Sativa seeds have been shown to be due to thymoquinone, the major component of the essential oil, which is also present in the fixed oil.

Swamy and Huat, (2003) [55]also have investigated the antitumor activity of α-hederin from N. sativa against LL/2 (Lewis Lung carcinoma) in BDF1 mice. Swamy and Huat (2003) observed that α-hederin also induced death of murine leukemia P388 cells by a dose- and time-dependent increase in apoptosis.

Badary et al., (2003) [56] proved the antioxidant properties of *Nigella Sativa* seeds.

Farah and Begum, (2003) [57] have examined aqueous and alcohol extracts of *N.Sativa in vitro* in inactivating MCF-7 breast cancer cells. objective of this study was to expose MCF-7 breast cancer cells to aqueous and alcohol extracts and in combination with H2O2 as an oxidative stressor. Measurement of cell survival under various concentrations and combinations was conducted using standard cell culture techniques, exposure protocols in 96 well plates and Fluoro-spectrosphotometry. Following cellular growth to 90% confluency, exposure to water (WE) and ethanol (AE) extracts of N. sativa and H2O2 was performed. Toxicity index (LC50) was calculated from percent survival using regression analysis. Results showed that the alcohol extract and its combinations were able to completely inactivate the MCF-7 cells (LC50 ranged from 377.16-573.79 in descending potency for H2O2 + AE, AE and Mix of WE and AE). H2O2 alone effectively inactivated MCF-7 cells (LC50 = 460.94)

Salim and Fukushima ., (2003)[58] demonstrated that the volatile oil of N. sativa has the ability to inhibit colon carcinogenesis of rats in the post-initiation stage, with no evident adverse side effects. Chemopreventive effects of orally administered Nigella sativa oil on the induction and development of 1,2-dimethylhydrazine-induced aberrant crypt foci (ACF), putative preneoplastic lesions for colon cancer, were investigated in Fischer 344 rats.

Also, Mabrouk, et al., (2002)[59] proved that supplementation of diet with honey and N. sativa has a protective effect against MNU (methylnitrosourea)-induced oxidative stress, inflammatory response and carcinogenesis in lung, skin and colon.

Ghosheh, et al., (1999)[60] described the quantification of four pharmacologically important components: thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ), and thymol (THY), in the oil of *N. sativa* seed by HPLC.

Tsunoda, et al., (1998)[61] investigated a colon cancer model in rats as an *in vivo* secondary screen for the general evaluation of new anticancancer agents against colorectal cancer.

Salomi et al., (1991)[62] reported that topical application of *N. sativa* extract inhibited two-stage initiation/promotion [dimethylbenz[a]anthracene (DMBA)/croton oil] skin carcinogenesis in mice. As well as intraperitoneal administration of N. sativa (100 mg/kg body wt) 30 days after subcutaneous administration of MCA (20-methylcholanthrene) inhibited soft tissue sarcomas to 33.3% compared with 100% in MCA-treated controls.

Salomi, et al., (1989)[63] have prepared an extract of *Smilax china, Hemidesmus indicus* and *Nigella Sativa* on the ratio 3:2:1, prepared by boiling in water. Cytotoxic studies with the three components showed activity in Nigella sativa at a concentration of 25 microgram equivalent of the dry powder against Dalton's lymphoma ascites cells. Animal experiments indicated the retarded growth of ascites as compared to the controls with a longivity of 90%.

CHAPTER III

3. AIM AND OBJECTIVES

The different types of cancer has most prevalence life threatening disease in the globe due to uncontrolled cell division and growth in affected area therefore badly affecting normal existence of life. Those who are affected with cancer can cure or reduce the symptoms with natural products.In india most of the peoples affected Colon cancer which included man 73% and woman 37%. Modern day chemotherapy treatment induced severe toxicities including weakening of body than before treatment of drugs.

Plant based medicines play an essential role in primary health care system. As well as importance of plant derived medicines are seriously under estimated in modern medicine. In recent years much attention has been shown therapeutic impact of plant derived drugs and phytochemicals on various infectious and non infectious diseases of cancer. Compared to it's alternative forms traditional medicines were practicing siddha, auyurveda and unnani but no scientific evaluation. The selection of this plant *Nigella Sativa* was made on the basis of literature survey to found the parameters such as:

- High therapeutic value
- Wide use in Indian Traditional System of Medicine
- Degree of research work which is not yet performed

So this work primarily aim to evaluate the anticancer activity of plant *Nigella sativa* stem extract on cell proliferation of colon carcinoma by using *invitro* and *invivo*.

CHAPTER IV

4. PLAN OF WORK

- Collection and authentication of plant *Nigella sativa*.
- Drying of plant stem
- Ethanolic extraction of dried stem by cold maceration.
- Preliminary phytochemical study of preapared *Nigella staiva* stem extract
- Acute toxicity study of Nigella Sativa stem extract
- Evaluation of *Invitro* cytotoxic activity
    - MTT Assay
- Study of *Invivo* cytotoxic activity.
    - Evaluation in Dimethyl Hydrazine (DMH) induced colorectal carcinoma in male wistar rats

CHAPTER V

5. PLANT PROFILE

Fig.No 5: *Nigella sativa*

5.1. Taxonomical classification [64]

Kingdom : *Plantae*

Sub kingdom : *Trachebionta*

Superdivision : *Spermatophyta*

Division : *Magnoliophyta*

Class : *Magnoliopsida*

Subclass : *Magnoliidae*

Order : *Ranunculaceae*

Genus : *Nigella.L*

Species : *Nigella sativa.L*

5.2. Description:

Annual herb,30-50 cm high, pubescent or glandular-hirsute. Leaves much divided, finely pinnate, leaf- segments linear to linear-lanceolate . Flowers solitary at end of branches, blue, star-shaped, sepals 5, petaloid, oval c.7-14x 6-8mm, shortly clawed; petals smaller than the sepals, 5(-8), nectariferous, with a bent claw and two lobes, stamens numerous, carpels 2-10 fused with five free styles. Fruit several follicles, crowned by persistent styles, many-seeded, brownish when ripe; seeds dark grey to black, trigonous, wrinkled , white and oleaginous inside, aromatic.

5.3.Habitat & Distribution:

The plant is found wild in southern Europe, northern Africa , Asia Minor and in the Mediterranean region, but has been cultivated into other parts of the world includingSaudi Arabia, Mediterranean countries, northern Africa and parts of Asia, in UAE rarely cultivated in private farms.

5.4.Table 2 :Vernacular Names

English	*Black-caraway, black-cumin, fennel-flower, nigella, nutmeg-flower*
Hindi	*Kalonji*
Bengali	*Mogrel*
Malayalam	*Karum Jiragam*
Marathi	*Kale Til*
Tamil	*Karum jeera*

5.5.*Nigella Sativa* seed Benefits[65]

It is reported to have the following medicinal benefits already

- Pain killing
- Antibacterial
- Anti inflammatory and Pro aptosis[66]
- Anti ulcer
- Anti cholinergic

- Anti Fungal
- Anti hypertensive
- Antispasmodic
- Anti viral
- Bronchodilator
- Anti Diabetic
- Liver protecting
- Hypotensive
- Insulin sensitizing
- Leukotriene antagonist
- Tumour necrosis factor
- Alpha inhibitor
- Anti oxidant

Nigella sativa seeds were used against Cough, Amenorrhoea, Thermogenic, Carminative, Diuretic, Emmenegogue, Anodye, Antibacterial, Antiinflammatory already.

CHAPTER VI

6. MATERIALS AND METHODS

6.1.Collection And Authentification Of Plant

The stem of *Nigella Sativa* was procured from the Botany Central council for Research in Ayurvedia and Siddha Govt of India and authenticated by Chelladurai.V research officer Botany Central council for Research in Ayurved and Siddha Govt of India. CERTIFICATE NO:UWAN/109/16

6.2.Extraction of Nigella Sativa Stem

Stem material was shade dried in room temperature for 3 days and then coarsely powdered.

Powdered plant material (500g) was defatted using hexane and defatted material was extracted with (2000 ml ×3) of methanol which was kept overnight (cold maceration) and filtered, filtrate was completely evaporated under reduced pressure using Rotavapor (MeBG) yield 13.6%w/w.

The spent material after ethanol extraction was extracted with distilled water (2000 ml×3) filtered and filtrate was completely dried under reduced pressure using Rotavapor (WtBG) yield 25.2% w/w.

The preliminary phytochemical study of defatted methanol extract indicated the presence of Anthraquinone glycosides, flavanoids, phenols, carbohydrates.

6.3.Phytochemical Screening[67]

6.3.1.Test for carbohydrates

Molisch's test: Dissolve small quantity of 300mg alcoholic and dried leaf extract of *Nigella Sativa* separately in 4ml distilled water and filter. The filtrate have to be subjected to Molisch's test.

Fehling's test: Dissolve a small portion of NS stem extract in water and treat with Fehling's solution.

6.3.2.Test for phenols

Spot NS stem extract on a filter paper. Add a drop of phosphomolybdic acid reagent to the spot and expose to ammonia vapours..

6.3.3.Test for flavanoids

Shinoda test**:** To 2 to 3ml of NS extract, a piece of magnesium ribbon and 1ml of concentrated HCl has to be added .

Lead acetate test: To 5ml of NS stem extract add 1ml of lead acetate solution..

6.3.4Test for tannins

Braemer's test**:** To a 2 to 3ml of NS stem extract,add 10% alcoholic ferric chloride solution..

6.3.5Test for steroid/terpenoid

Liebermann-Burchardt test**:** To 1ml of *Nigella Sativa* stem extract, add 1ml of chloroform, 2 to3ml of acetic anhydride and 1 to 2 drops of concentrated Sulphuric acid. If Dark green coloration of the solution takes place it indicates the presence of steroids and dark pink or red coloration of the solution indicates the presence of terpenoids.

6.3.5. Test for alkaloids

Draggendorf's test**:** A drop *Nigella Sativa* stem extract was spotted on a small piece of precoated TLC plate and the plate was sprayed with modified Draggendorf's reagent.

Hager's test**:** Treat the *Nigella Sativa* stem extract with few ml of Hager's reagent.

Wagner's test**:** Add few ml of Wagner's reagent to *Nigella Sativa* stem extract..

6.3.6.Tests for Glycosides

Legal's test: Dissolved the *Nigella Sativa* stem extract [0.1g] in pyridine [2ml], add sodium nitroprusside solution [2ml] and made alkaline with Sodium hydroxide solution.

6.3.7.Test for Saponins

Foam test: 1ml of *Nigella Sativa* stem extract was dilute with 20ml of distilled water and shaken with a graduated cylinder for 15 minutes

6.3.8.Test for Anthraquinones

Borntrager's test: Add about 50 mg of powdered *Nigella Sativa* stem extract withd with 10% ferric chloride solution and 1ml of concentrated HCl. The extract has to be cooled, filtered and shake the filtrate with diethyl ether. The ether extract further extraction with strong ammonia .

6.3.9.Test for Amino acids

Ninhydrin test: Dissolve a small quantity of the *Nigella Sativa* stem extract in few ml of water and adde 1ml of ninhydrin reagent.

6.3.10.Test for fixed oils and fats

Press small quantity of the *Nigella Sativa* stem extract between two filter paper.

6.4. *invitro* Anticancer activity

6.4.1.MTT assay[68]

Reagents

PBS
MTT (5 mg/ml in PBS) – filter and keep dark, prepare freshly
Acidic isopropanol (0.1N HCl in absolute isopropanol)
96-well plate (flat bottom)

Protocol

MTT Assay

1. For adherent cells, remove the medium and replace it with 100 µL of fresh culture medium. For non-adherent cells, centrifuge the micro-plate, pellet the cells, carefully remove as much medium as possible and replace it with 100 µL of fresh medium.
2. Add 10 µL of the 12 mM MTT stock solution (prepared in step 1.1) to each well. Include a negative control of 10 µL of the MTT stock solution added to 100 µL of medium alone.
3. Incubate at 37°C for 4 hours. At high cell densities (>100,000 cells per well) the incubation time can be shortened to 2 hours.
4. Add 100 µL of the SDS-HCl solution (prepared in step 1.2) to each well and mix thoroughly using the pipette.
5. Incubate the microplate at 37°C for 4– hours in a humidified chamber. Longer incubations will decrease the sensitivity of the assay.
6. Mix each sample again using a pipette and read absorbance at 570 nm.

Percentage inhibition of the extract against all cell line was calculated using the following
formula.

% of cell survival = $(\mathbf{At} - \mathbf{Ab}) \div (\mathbf{Ac} - \mathbf{Ab})$

At = Absorbance of Test,

Ab= Absorbance of Blank (Media),

Ac= Absorbance of control (cells)

% cell inhibition = 100 - % cell survival

The effects of extracts were expressed by IC50 values calculated from dose response curves.

6.5. Acute toxicity study

The acute oral toxicity study was to be conducted in compliance with OECD guideline 423, Animals were fasted overnight (~12 h) and weighed. Test doses of *Nigella Sativa* stem ethanolic extract were calculated in relation to the body weight of every fasted animal, and administered via oral gavage at 2000 mg/kg. The animals were regularly and individually observed for behavioral changes and general toxicity signs after dosing for the first 24 h, with special attention being given during the first 4 h. Thereafter, observation was continued daily for a total of 14 days.Mortality was Nil. But observed some behavioural changes and writhing response and loose stools at the dose of 2000mg/kg. Hence 1/5th and 1/10th dose of 2000mg/kg i.e.; 200mg/kg and 400mg/kg have been used

Table .No:3.

Group	Dose(mg/kg)
Group I	5
Group II	50
Group III	500
Group IV	2000

6.6.Evaluation of *invivo* anti colon cancer activity

6.6.1.Test animal

Male wistar rats of 6-8weeks old

6.6.2.Housing

The experimental animal room temperature maintained at 22°C±3°C OECD guideline-425, 2001. These ranges are designed to allow homeotherms to maintain metabolic rate or to be within their thermo neutral zones. Because, temperature below the recommended range leads to increased food

intake, increased energy expenditure but decrease in efficiency. In contrast, temperature above the recommended range leads to decreased food.

6.6.3.Humidity

The relative humidity maintained at 40%-60% preferably not exceeds 70% (OECD-425, 2001).The relative humidity below the recommended range can develop lesions such as ring tail and food consumption may be increased take.

6.6.4.Light

12-12 hours, Light/dark cycle. Appropriate lighting and light cycle play a key role in maintaining the physiology and the behavior rat. Light provided for adequate vision and for neuroendocrine regulation of diurnal and circadian cycles (CPCSEA guidelines for laboratory animal facility 2003).

6.6.5.Caging

Polypropylene cages with solid bottom and walls. Lids made up of stainless steel grill capable of holding of both feed and water. 4 to 5 is put per cage.

6.6.6.Feeding condition

Sterile laboratory feed (*ad libitum*) and RO water bottles daily.
Feed – Brown colored chow diet.

6.6.7.Drug administration

Animals wre normally provided with food ad libitum. And weighed prior to study. In these theree groups Group 1 served as normal control which received vehicle(gum acasia suspension),Group 2 prepared as disease control were given intra peritoneal injection of DMH twice a week for two consecutive weeks at a dose of 30 mg/kg body weight. And the group 3 where the same aforementioned method used to induce colorectal cancer treated with Nigella Sativa stem extract at a dose of 200mg/kg body weight daily for total 30 days of study period by oral route.

6.6.8.Clinical observations

All rats were monitored continuously for 4 hour after dosing for signs of toxicity. For the remainder of the 14 days study period, animals were monitored and any additional behavioral or clinical signs of toxicity. Animal's body weight was measured prior to dosing and on days 7 and 14.On all animals were killed and at the end of the study LD50 value was established. Clinical observations and gross pathological examination was carried out.

6.6.9.DMH induced colon cancer

6.6.9.1. Animals

Healthy adult male wistar rats 18 numberes are procured and assigned to three groups. In these theree groups Group 1 served as normal control which received vehicle(gum acasia suspension),Group 2 prepared as disease control were given intra peritoneal injection of DMH twice a week for two consecutive weeks at a dose of 30 mg/kg body weight. And the group 3 and 4 where the same aforementioned method used to induce colorectal cancer treated with Nigella Sativa Stem extract at a dose of 200mg/kg and 400 mg/kg body weight daily for total 30 days of study period by oral route. The animals were kept in polypropylene cages (4 per cage) and fed standard pellet diet for 1 week. Thereafter, the animals were randomly divided into three groups each containing 6 rats and maintained under controlled conditions of temperature (24 ± 2 °C), humidity (50 ± 10%), and 12-h light/dark cycle and tap water was provided *ad libitum.*

DMH had to be dissolved in 1mM EDTA just before use and pH was adjusted with NaOH to confirm the stability of carcinogen [69]**.** Rats of Group 2 & 3 are given with intra peritoneal injections of DMH twice a week for 2 weeks at a dose of 20 mg/kg of body weight.

6.6.9.2. Table No 4 : Invivo anti-cancer study – Animal grouping

Group	Treatment
1	Normal control which receive vehicle
2	Disease control where 30 m/kg of i.p injection of DMH given twice for 2 consecutive weeks.
3	Treated group where DMH induced colon cancer symptoms were treated with prepared extract at a dose of 200 mg/kg body weight for 30 days
4	Treated group Where DMH induced induced colon cancer symptoms were treated with prepared extract at a dose of 400 mg/kg body weight.

6.6.9.3.Body weight changes

The body weight changes of the control, DMH and *nigella sativa* stem extract treated rats were measured throughout the study. The rats were weighed at the beginning of the experiment and then subsequently once a week and finally before sacrifice.

6.6.9.4.Determination of aberrant cryptic foci

At the end of the study, rat colons were removed and flushed with potassium phosphate buffered saline (0.1 M, pH 7.2). Colons were split open longitudinally and placed on strips of filter paper with their luminal surfaces open and exposed. Another strip of filter paper was placed on top of the luminal surface. The colons were then secured and fixed in a tray containing 10% buffered formalin overnight. Each of the fixed colons was cut into proximal and distal portions of equal lengths and each portion was further cut into 2 cm long segments. Each segment was placed in a petridish and stained with 0.2% methylene blue solution for 2 min. The segments were examined using a light microscope at low magnification to score the total number of ACF as well as the number of crypts per focus. ACF were distinguished from normal crypts by their thicker, darker-stained, raised walls with elongated slit-like lumens and significantly increased distance from the lamina to basal surface of cells.[69]

6.6.9.5. Apoptosis measurement in colonic mucosa

Apoptosis evaluation was carried out in paraffin-embedded sections of normal colonic mucosa and tumours stained with haematoxylin–eosin.[70] At least 20 full longitudinal crypt sections of normal mucosa/rat were scored at the microscope, determining the presence of cells in each crypt with the following characteristics of apoptosis: cell shrinkage, loss of normal contact with the adjacent cells of the crypt, chromatin condensation or formation of round or oval nuclear fragments ("apoptotic bodies"). When clusters of more than one apoptotic body were seen within the diameter of one cell, these bodies were considered as fragments of one apoptotic cell. Tumour apoptosis was determined by scoring at least 1000 cells/rat for the presence of apoptotic cells that were coded as described above.

apoptotic index (AI) = Number of apoptotic cells/cells scored × 100).

6.6.9.6. Colon crypt Histopathology

Necropsy was performed for all rats sacrificed.
Entire intestine was removed and large intestine portion is removed.
Which were cut opened longitudinally.

Then it is taken for wash with PBS.

Mucosal surface lesions are noted and that portions are taken.

Fixation performed by 4%V/V formalin in PBS overnight.

Each segment was placed in a petridish and stained with 0.2% methylene blue solution for 2 min. The segments were examined using a light microscope at low magnification to score the total number of ACF as well as the number of crypts per focus.

ACF were distinguished from normal crypts by their thicker, darker-stained, raised walls with elongated slit-like lumens and significantly increased distance from the lamina to basal surface of cells. ACFs in the colon were counted as described by Bird

6.6.9.7. Haematological parametres

Blood samples were collected for haematological analysis in Vacutainer tubes with 1.5% EDTA and differentially quantified through a Coulter T890 for the following: WBC, RBC and platelet counts and hemoglobin determination. During the course of the study, the haematological parameters of the animals were tracked on every 5^{th} day. For this, blood was collected from the caudal vein into heparinised tubes and total WBC count and haemoglobin level were checked.

6.6.9.7.1. WBC Count

The whole blood was diluted using a diluent which haemolyses red cells, leaving all the nucleated cells intact. The number of white cells in a known volume and known dilution were counted using a counting chamber.

0.02 ml of blood was added to 0.38 ml of diluting fluid and mixed well. The diluted blood was charged into a Neubauer counting chamber. After 3-4 min, the total number of white blood cells in the four large corner square chambers was counted.

$$\text{Total WBC} = (\text{Number of cells counted} \times 50) / mm^3$$

6.6.9.7.2.Determination of haemoglobin (Hb) content:

Cyanmethaemoglobin method

Haemoglobin was treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate. The ferricyanide forms methaemoglobin, which is converted to cyanmethaemoglobin by cyanide. The intensity of colour formed is measured at 546 nm against blank. The optical density is directly proportional amount of hemoglobin.

Procedure:

0.02 ml of fresh whole blood was mixed with 5 ml of the cyanmeth reagent. The optical density was measured at 546 nm against blank after 5 min

incubation at room temperature. The OD of standard solution corresponding to 60 mg/dl haemoglobin at 546 nm was also read against reagent blank.

Haemoglobin (g/dL) = (OD of treated x 60 x 0.251) / OD of standard

During necropsy, the liver, kidneys, colon, spleen, heart, pancreas, stomach, lungs were removed, and weighed[71]

Statistical analysis

Values are given as means ± SD of each group. Data were analyzed by one-way ANOVA. The results are considered statistically significant at $P < 0.05$.

CHAPTER VII

7. RESULTS AND DISCUSSION

7.1. Extraction of *Nigella sativa* stem

Percentage yield of Nigella Sativa stem extract by cold maceration was found to be 10.2% w/w.

Table.No: 5 Extraction of *Nigella Sativa* stem extract

Plant name	Part using	Method of extraction	Solvents	Yield in per-centage
Nigella sati-va	Stem	maceration	ethanol	20.3

7.2.Preliminary phyto chemical screening

Niglla sativa stem extract were subjected to various chemical tests for identification and reported.

Molisch's test- indicated Formation of reddish brown ring indicatng the presence of carbohydrates.

Fehling's test- brown color indicating the presence of carbohydrate.

Test for Phenols- Blue coloration of the spot indicates the presence of phenols A pink or red coloration of the solution indicates the presence of flavonoids in the drug.

Shinoda test- Flocculent white precipitate indicated the presence of flavonoids.

Braemer's test- Dark blue or greenish grey coloration present indicates the presence of tannins in the drug.

Libermann Buchard test - Dark green coloration of the solution takes place it indicates the presence of steroids and dark pink or red coloration of the solution indicatesd the presence of terpenoids.

Dragendroff's test - Orange coloration of the spot indicated the presence of alkaloids.

Hager,s Test- Yellow precipitation indicated the presence of alkaloids

Wagner's test- The reddish brown precipitation indicated the presence of alkaloids

Legal's Test - Pink to red color solution indicated the presence of glycosides.

Foam test - A 1cm layer of foam formation indicated the presence of Saponins

Borntrager's Test- Pink or red coloration of aqueous layer indicated the presence of Anthraquinones

Ninhydrin Test- Blue color indicated the presence of amino acids.

Test for fixed oils- Oil stains on the paper indicated the presence of fixed oils.

Table No 6 : Preliminary phytochemical screening of Nigella Sativa Stem extract

Class of Compounds	Tests performed	Results
Carbohydrate	Molisch's Test Fehling's test	-
Phenols	Phosphomolybidic test	+
Flavanoids	Shinoda test Lead acetate test	+
Tannins	Braemer's test	-
Alkaloids	Wagner's Mayer's Draggendrof's test	-
Glycosides	Legal's test Brontranger's test	+
Saponins	Foam test	+
Sterols	Salkowski's test	-
Amino Acids	Ninhydrin test	-
Terpenoids	Libermann Burchardt test	-

NB:

-indicate not present

+ indicate presence

7.3 Acute toxicty study

The animals were regularly and individually observed for behavioral changes and general toxicity signs after dosing for the first 24 h, with special attention being given during the first 4 h. Thereafter, observation was continued daily for a total of 14 days.Mortality was Nil. But observed some behavioural changes and writhing response and loose stools at the dose of 2000mg/kg. Hence 1/5th and 1/10th dose of 2000mg/kg

i.e.; 200mg/kg and 400mg/kg have been used.

Table.No.7:Changes in wellness parameters during acute toxicity study

	Group 1 (5mg/kg)	**Group 2(50 mg/kg)**	**Group 4(500 mg/kg)**	**Group 5 (2000mg/kg)**
Alertness	Normal	Normal	Normal	Absent
Anxiety	Normal	Normal	Normal	Normal
Roaming	Normal	Normal	Normal	Normal
Depression	Normal	Normal	Normal	Normal
Writhing	Absent	Absent	Absent	Present
Defaecation	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal
Diarrhoea	Absent	Absent	Absent	Present
Sleep	Normal	Normal	Normal	Abnormal

7.4.*Invitro* Anticancer Activity

7.4.1. MTT assay

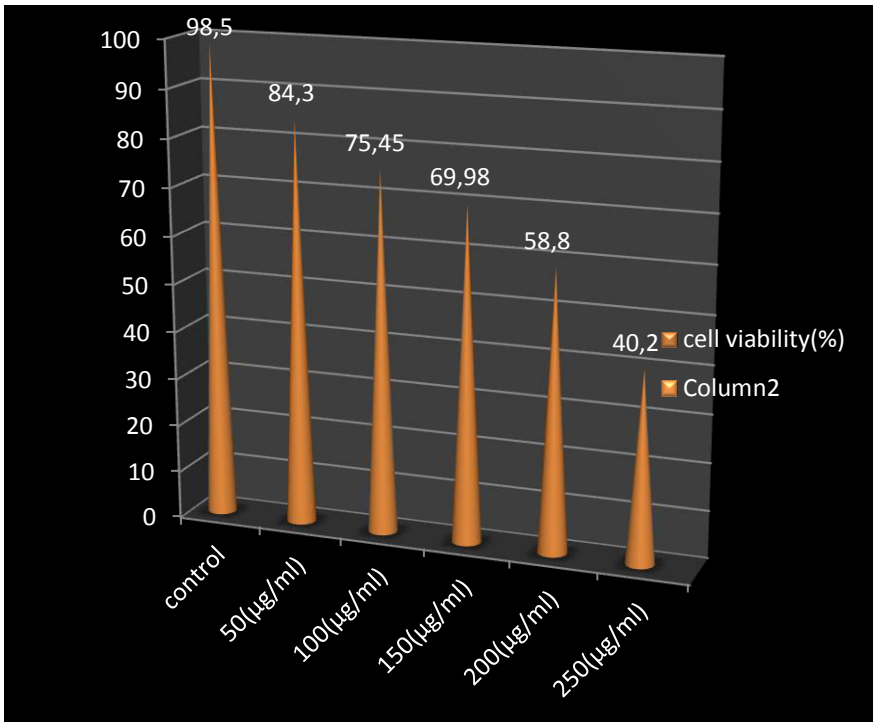
This is a colorimetric assay where we measure the reduction of yellow3-(4,5 dimethyl thiazole-2-yl-)2-5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. When MTT get inside the cells and passes to mitochondria ,where it is reduced to an insoluble coloured Formazan product. Reduction of MTT only occurs when cells are metabolically active an the level of activity is measured by presence of formazan spectrophotometrically.

Table No 8 : Invitro cytotoxic activity (MTT ASSAY)

NS stem extract Sample Concentration (µg/ml)	MTT assay % viability
Control	98.50±0.35
50	84.30±0.21
100	75.45±0.26
150	69.98±0.24
200	58.80±0.29
250	50.20±0.30

Graph

MTT assay



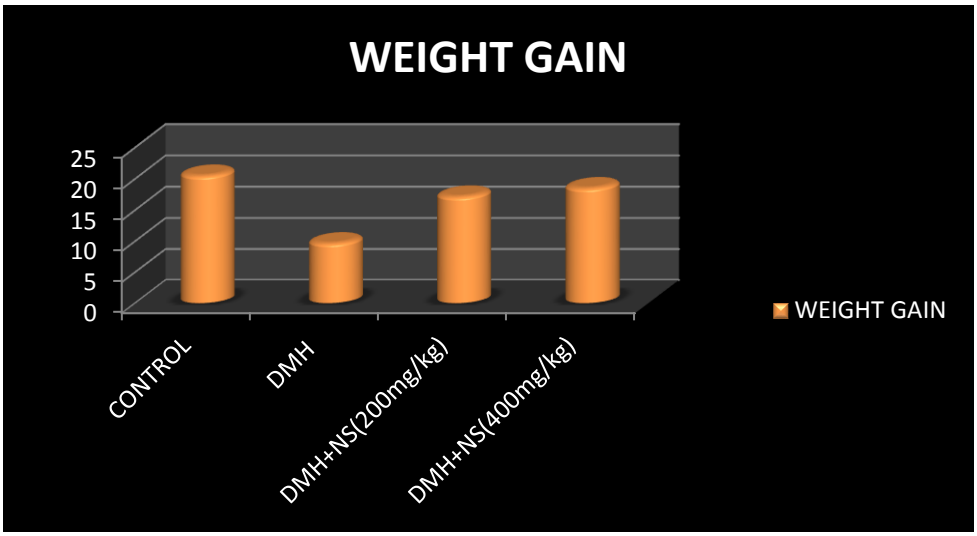
7.5.Body weight changes

During the experimental period 30 days, the carcinogen-exposed rats (Group 2) exhibited a significantly (p< 0.05) low gain in body weight and a low growth rate throughout the experimental period as compared to Groups 1 and 3Oral administration of *nigella sativa* stem extract at a dose of 200 mg/kg b.w resulted in a significant improvement in weight gain relative to treatment with DMH alone. Values are mean ± SD, n=6 in each group, statistically significant **p<0.01,*p<0.05 when compared with disease control (DMH treated) group. Values are mean ± SD, n=6 in each group, statistically significant a**p<0.01, b*p<0.05 when compared with disease control (DMH treated) group.

Table No 9 : Body weight changes on treatment with ethanolic extract of *Nigella sativa* stem

Group	Initial weight(average) in grams	Final weight(avera gms	Weight gain
Control	137.83 ± 15.68	158.33±9.66	20.5±10.634**
DMH treated	166.16±18.17	175.33±21.64	9.66±4.082
DMH+NS stem extract treat-ed(200 mg/kg)	169.16±26.91	186.3±27.688	17.16±7.08*
DMH+NS stem extract treat-ed(400mg/kg)	149±25.16	167±14.56	18.5±5.08*

Graph



7.6. Aberrant crypt foci scoring

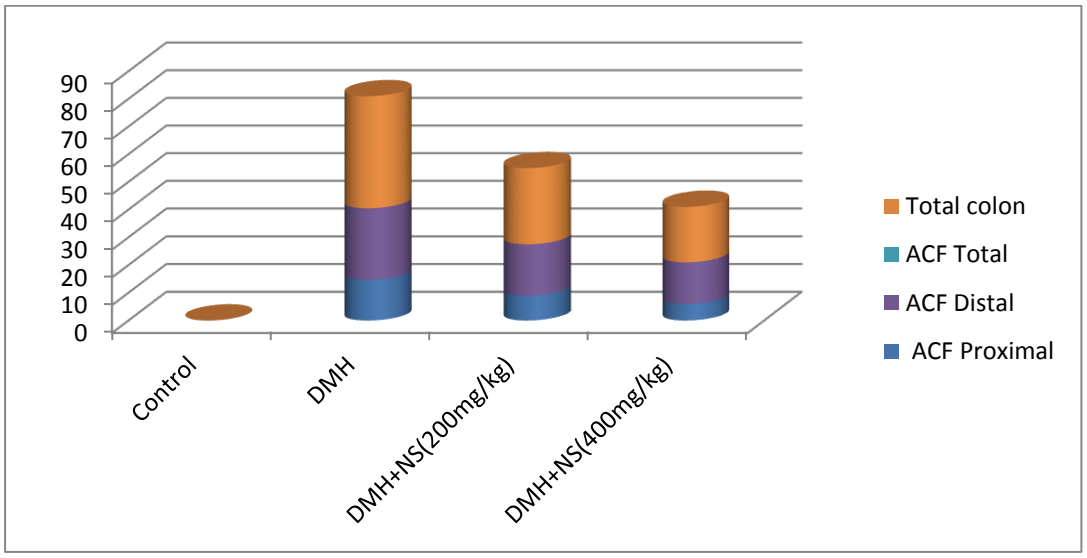
ACF formation was observed in all DMH induced groups. The majority of ACF appeared in the distal colon of the rats injected with DMH. Oral administration of Nigella sativa stem extract at 200 mg/kg b.w. could slightly inhibit the formation as well as the total number of ACF , as compared to rats injected with DMH alone , but there was no reduction in number of aberrant crypt. Values are mean ±SD, n=6 in each group, statistically significant **p<0.01,*p<0.05 when compared with disease control (DMH treated) group. Values are mean ±SD, n=6 in each group, statistically significant a**p<0.01 when compared with disease control (DMH treated) group.

Table .No: 10 : Distribution of altered aberrant crypt foci (ACF) category in proximal, distal and total colon of rats exposed to DMH and *Nigella sativa* stem extract.

Group	Animal Nos	Drug	Dose (Mg/kg) & routemof adminstration	Total num-ber of ACF
Proximal colon				
Control	6	vehicle	Oral	0
DMH	6	DMH	30mg/kg i.p	14.66±2.33
DMH&NSSE(200 mg/kg)	6	DMH+NSSE	30mg/kg i.p + 200 mg/kg oral	9± 3.1**
DMH&NSSE(400mg/kg)	6	DMH+NSSE	30mg/kg i.p + 400 mg/kg oral	6±1.53**
Distal colon				
Control	6	Vehicle	Oral	0
DMH	6	DMH	30mg/kg i.p	25.83±4.3
DMH&NSSE(200mg/kg) (400mg/kg)	6	DMH+NSSE	30mg/kg i.p + 200 mg/kg oral	18.5±3.08**
DMH&NSSE(400mg/kg) (400mg/kg)	6	DMH+NSSE	30mg/kg i.p + 400 mg/kg oral	15±2.33**
Total colon				
Control	6	VEHICLE	Oral	0
DMH	6	DMH	30mg/kg i.p	40.49±6.3
DMH&NS treat-ed(200mg/kg)	6	DMH+NSSE	30mg/kg i.p + 200 mg/kg oral	27.5±6.18**
DMH&NSSE(400mg/kg)	6	DMH+NSSE	30mg/kg i.p + 400 mg/kg	20±5.13**

Graph

ACF Scoring



7.7.Apoptosis Scoring

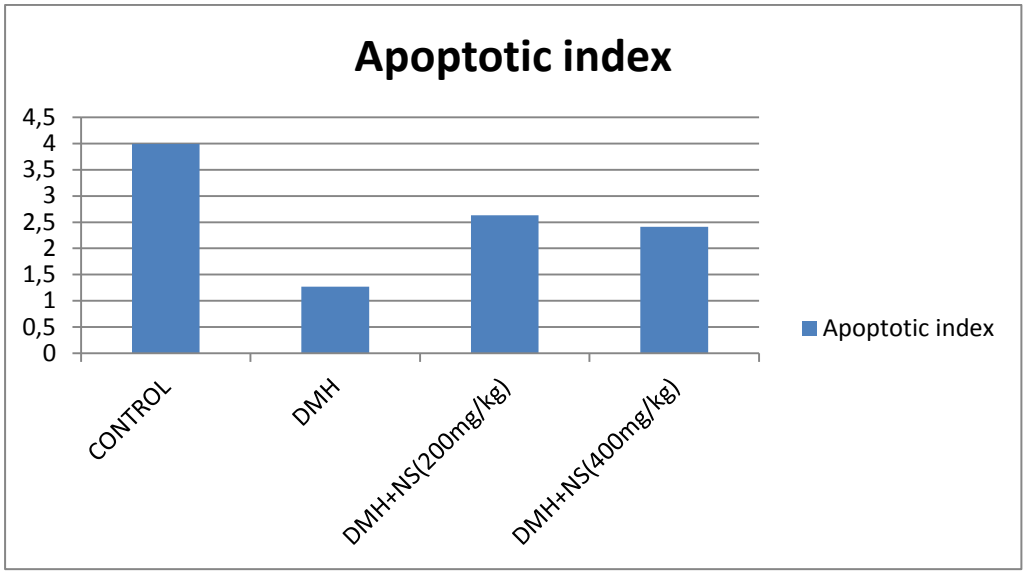
The apoptosis index (AI %) was estimated as the percentage of apoptotic cells among the total number of counted cells in a whole colonic crypt. The apoptotic index was increased in groups treated with DMH+NS extract compared to only DMH treated group. Values are mean ±SD, n=6 in each group, statistically significant **p<0.01when compared with disease control (DMH treated) group

Table.No:11 : Apoptosis Scoring

Effect of *Nigella Sativa* and DMH on apoptotic indexes.

Group	Apoptosis index
Control	3.53±0.46***
DMH	0.9±0.37
DMH+nigella sativa stem extract(200mg/kg)	2±0.63**
DMH+NSSE(400mg/kg)	2.41±0.52**

Graph

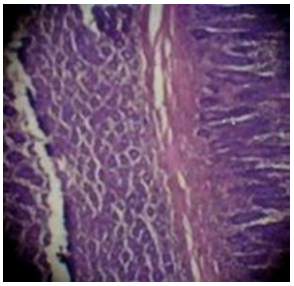


7.8.Histopathology of Colon crypt

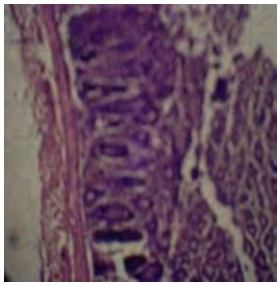
In control group of animals normal crypt can be viewed . But in DMH treated animals it shows thickened mucosa with densely packed inflammatory cell infiltration and hyperplasia. In third group where DMH induced cryptic foci were treated with 200mg/kg b.w Nigella sativa stem extract showed thickened mucosa in some got scattered.

Histopathology of colon crypt

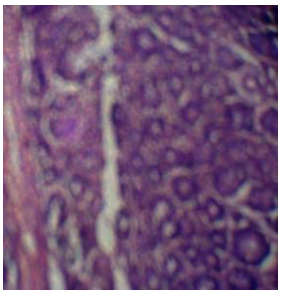
Fig. No: 9



Control



DMH



DMH+NSSE **(**200mg/kg)

DMH+NSSE**(**400mg/kg)

7.9. Haematological parameters

Values are mean±SD, n=6 in each group, statistically not significant p>0.05, when compared with disease control (DMH treated) group. There couldn't find any significant changes in haemetological parametres before and after treatment.

Table No 12 : Effect of Nigella Sativa stem extract and DMH on various haematological parameters.

Haemotological parameters	Change in haemotological parameters after induction							
	Control		DMH		DMH+NS Extract		DMH+NS Extract	
	Before	after	Before	After	Before	After	Before	After
WBC (cells/µL×103	9.1±1.7	9.97±2.4	6.06±1.89	8.53±2.42	10.2±3.41	10.56±4.27	9.23±2.42	90.12±2.2
RBC (cells/µL×106	6.5±0.05	7.10±0.26	4.24±1	5.09±1	5.12±1.04	6.5±0.69	6.23±2.1	4.5±2.41
HGB(g/dL)	11.3±1.8	11.9±1.04	11.1±2.32	11.9±2.04	9.4±2.51	12.6±1.41	9.5±5.1	9.3±2.1
PLT (cells/µL×103)	256±57	291±510	195±97	222±81.3	243.3±87.7	272±36	256±50	280±30

7.10.Relative weight of organs

Values are mean±SD, n=6 in each group, statistically not significant p>0.05, when compared with disease control (DMH treated)group. Relative weight of various organs when noted couldn't find any variations before and after treatment.

Table No 13: Effect of *Nigella Sativa* stem extract and DMH on relative weight of organs

Relative weight of organs(weight of organ/100)	Group			
	Control	DMH	DMH+NS stem extract	DMH+NS stem extract(400mg/kg)
Liver	3.369±0.33	3.69±0.22	3.11±2.56	3.4±2.56
Kidney	0.826±0.07	0.83±0.19	0.79±0.07	0.81±0.07
Heart	0.413±0.04	0.382±0.09	0.24±0.065	0.45±0.084
Lungs	0.859±0.1	0.748±0.0299	0.5±0.084	0.62±0.04
Pancreas	0.04±0.06	0.391±0.165	0.47±0.09	0.49±0.08

The increasing rate of colorectal cancer has brought an conclusion about the role of change in food habitats and adoption of western life style in neoplasm [72] . Death due to colorectal cancer have reported due to late diagnosis and treatment.

As we discussed earlier since “prevention is better than cure” I have focussed my work on chemoprevention which aim at reducing the intensity of incidence of cancer with herbal remedies.

Hydrazine and its derivatives like DMH are such chemicals that are shown to be carcinogenic and mutagenic [73] DMH, an alkylating agent, when injected intraperitonially, is transported to the liver where it undergoes dehydrogenation and is converted to an active carbonium ion through several processes, to be excreted in the bile, where it mediates its carcinogenic activities on the mucosa while passing through the digestive tract.[74]

Aberrant crypts at colon are the earliest identifiable indication for colon cancer. These crypts appear by an increased size thicker and deeply stained epithelial lining and increased pericryptal zones.[75] They are putative neoplastic lesions of the colon.Budding and branching process, of crypts with outward pushed cells are known as crypt fission, which forms larger sized foci over time[76]. They appear within two weeks after carcinogen injectionas single crypts that expand by crypt branching or multiplication. ACF containing four or more crypts is known to correspond to the promotion stage of colon carcinogenesis

This study performed the evaluation of anticancer effect of a *Nigella sativa* stem extract both *invivo* and *invitro* for colon cancer.

Phytochemical screening of *Nigella sativa* stem extract shows the presence of phenols,saponins,and flavanoids.

Invitro cytotoxic study of *nigella sativa* stem extract performed by MTT assay. Which indicated a decrease in cell viability with increased dose of extract of *Nigella Sativa* stem. It is specific to colonic epithelium as a result of incomplete repair of DNA in the colon compared to other organ tissues.

In invivo screening performed by DMH induced colorectal changes.DMH is a colon carcinogen.

Results of invivo pharmacological screening shows that oral administration of NS stem extract brought a reduction in ACF to a small extent in comparison with normal control and DMH only treated group.

There were no significant changes in the relative weight of individual organs among the groups and also in haematological parameters. Histology of The results of the present study demonstrated that administration of ethanolic extract of *Nigella sativa* stem at a dose of 200mg/kg body weight during either the initiation, post-initiation or entire period phase could bring slight inhibition DMH-induced colon carcinogenesis in rats.

A common feature of GI tumours is weight loss which shows aggressiveness of the disease. Similiarly DMH treated groups shown reduction in

weight gain compared to normal control rats.In this study colon were examined for ACF 30days after the first injection of DMH i.p. Crypts were observed at proximal and distal colon.

Herb based drug industry is growing day by day. The destruction of natural habitats over exploitation and unsustainable harvest has lead to a severe scarcity of raw material. These problems have adversely affected the quantity of herbal drugs.

In this study we used DMH as colon cancer inducer and ethanolic extract of stem of Nigella Sativa as the test drug. The purpose of this investigation, which is a part of a large-scale study to prove the inhibitory effect of *Nigella Sativa* on colon cancer using established colon cancer model.

CHAPTER VIII

8. SUMMARY AND CONCLUSION

The current work revealed that the *Nigella sativa* stem shows anti-cancer activity comapred to normal. High light of this study is extraction with methanol and it's phytochemical screening. Phytochemical study shows the presence of phenols, flavanoids, saponins ,and glycosides. *Invivo* study of Nigella Sativa stem extract was performed in DMH induced colon carcinogenesis. The results of present study showed that the supplementation of Nigella sativa to the diets of rats for 28 days did not change the haemetological parameters as well as histo-pathological examinations which illustrated normal architecture of liver and heart after treatment. It's proved by no significant changes of serum ALT and AST level in treatment group compare to the control group. Absence of pathological condition of liver tissue in histological evaluation confirmed the result. This study also found that body weights of the rats in all groups are maintained during the experiment which indicating healthy status of animals. Invitro evaluation performed by MTT assay shows decreased cell viability with increasing concentrations of extract being cytotoxic. The identification of chemical compounds present in the stem of *Nigella sativa* stem which are responsible for the specific activity is remaining.

CHAPTER IX

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